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Identification and Purification of Metalloprotease from Dry Grass Pea (*Lathyrus sativus* L.) Seeds

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Abstract Proteolytic enzymes play a central role in the biochemical mechanism of germination. The present study reported the presence of Zn^{2+}-dependent endoproteases in the dry seeds of grass pea (*Lathyrus sativus* L.) with maximum caseinolytic activity observed at pH 8.0. Studies with class-specific inhibitors (specific for cysteine, serine, aspartate, and metalloproteases) on crude extract identified the inhibitory effect of 1,10-phenanthroline. This inhibitory effect was overcome by addition of Zn^{2+}, not with Fe, Ca, Cu, Mg, or Co and indicates that the protease is Zn^{2+} dependent. This metalloprotease was further characterized by attempting gelatin-PAGE zymography and observed three distinct zones of proteolytic activity with higher mobility. The protease fraction consisted of three isoforms as evidenced by the appearance of three different bands on gelatin-PAGE zymogram. We also purified these proteases to 110-fold by a three-step procedure comprising crude extract from dry seeds, $(NH_4)_2SO_4$ fractionation, and casein–alginate affinity chromatography. The molecular mass of isoforms of metalloproteases is 25, 18, and 14 kDa.

Keywords Metalloprotease · Purification · Partial characterization · Legume seeds · Grass pea

Introduction

Proteolytic enzymes play a central role in various biochemical mechanisms and are used in different objectives as in vitro and in vivo. Commercially, they are extremely important as more than 60% of the total enzyme market is made up of proteases; they are isolated from

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plants, animals, bacteria, and fungi. In recent years, proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity, active in wide range of pH, temperature, and in the presence of organic compounds as well as other additives [1–3]. Search for valuable proteases with distinct specificity is always a continuous challenge for varied industrial applications. Therefore, plant protease may turn out to be an efficient choice in pharmaceutical, medicinal, food, and biotechnology industry.

The plant seed is not only an organ of propagation and dispersal but also the major plant tissue harvested by humankind. The amount of protein present in seeds varies from ~10% (in cereals) to ~40% (in legumes), forming a major source of dietary protein. Proteolytic enzymes are intricately involved in many aspects of plant physiology and development [2, 4]. Numerous reports including our previous data supported that the proteases are responsible for protein degradation [5–10]. With renewed interest, there has been proliferation of reports in the last decade concerning purification and characterization of these proteases from leguminous and nonleguminous seeds [2, 11–16]. Exploration for existence of valuable proteases as well as understanding the appropriate physiological role of such proteases in plants is still an open area of investigation.

Lathyrus sativus is a legume (family Fabaceae) commonly grown for human consumption and livestock feed in Asia and East Africa. Like other grain legumes, *L. sativus* produces a high-protein seed. In earlier studies, Rajyalakshmi [17] reported the presence of high activity of metalloprotease in dry seeds and declined these activities in the later stages of germination. In dry seeds, the existence of cysteine and aspartic proteases has been demonstrated, whereas in germinating seeds, mainly cysteine proteases are found [4, 18]. The metalloproteases are apparently also important players in protein solubilization, although their contributions have scarcely been examined. Several classes of metalloproteases have been described in humans, bacteria, snake venoms, and insects. However, the presence and characterization of plant metalloproteases have rarely been described in the literature [2, 3, 19–22]. In the present study, we reported the purification and characterization of metalloprotease from the dry seeds of grass pea (*Lathyrus sativus* L.) seeds. It is shown that the presence of three isoforms of metalloproteases specifically requires zinc for their activity.

Materials and Methods

Grass pea (*Lathyrus sativus* L.) seeds were procured from local agricultural farm, Borbatla village, Ranga Reddy district, Andhra Pradesh. Healthy seeds of uniform size and weight were sorted and stored in a sterile plastic container until use.

Preparation of Dry Seed Extract

The dry seeds were ground thoroughly in a prechilled mortar with chilled 0.1 M Tris–HCl buffer pH 8.0. The extract was filtered and centrifuged at 10,000 rpm for 15 min. The supernatant was used for the estimation of proteins and assay of proteolytic enzymes.

Estimation of Proteins

Protein content in the seed extract was estimated by the method of Lowry [23]. The results were expressed as milligrams per gram seeds.

Assay of Proteolytic Enzyme

Endopeptidase activity was measured by the modified method of Beevers [24] using casein as substrate. The reaction mixture containing 1 ml of diluted enzyme extract, 1 ml of 1% casein (prepared in 0.1 N NaOH, pH adjusted to 7.0 with 0.1 N HCl), and 1 ml of 0.1 M Tris–HCl buffer pH 8.0. Incubation was carried out for 1 h at 40°C. The reaction was arrested by adding 1 ml of 20% trichloroacetic acid. The contents of the tube were kept at 4°C for 15 min and centrifuged at 3,000 rpm for 15 min. An aliquot of the supernatant was used for the determination of amino acids by ninhydrin method [25]. The results were expressed as micromoles of amino acids released per hour in 1 g of seed under experimental conditions.

Purification of an Acidic Protease

A procedure for the purification of metalloprotease from grass pea seeds has been standardized. Starting with dry seeds, the following steps were carried out at 4°C. (1) 10 g of dry seeds homogenized in 0.1 M Tris–HCl buffer pH 8.0. A clear homogenate was obtained after centrifugation at 10,000 ×*g* for 15 min. (2) Dry seed extract was concentrated by ammonium sulfate fractionation and the 20–40% saturated fraction, dissolved in minimum volume buffer and dialyzed extensively with 0.1 M Tris–HCl buffer pH 8.0 and used for the estimation of proteins and assay of proteolytic enzymes. (3) The dialyzed protein from ammonium sulfate was subjected to pass through an affinity column of casein–alginate. The protein elution pattern KCl linear gradient was monitored spectrophotometrically at 280 nm, and fractions containing enzyme with high specific activity were pooled. (4) Pooled sample was again subjected to gel filtration on Sephadex G-50 column (1 cm×15 cm) and eluted with buffer. The eluted fractions were used for protein and enzyme assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the proteins at each step of purification was carried out by Laemmli [26]. All the enzyme activity recovered in the fractions were concentrated by lyophilization and stored.

Statistical Analysis

Each value presented in tables represents the arithmetic mean±SE of five independent determinations, unless otherwise stated. The level of significance was calculated by *t* test.

Results

The extracts of dry grass pea seeds showed the presence of high amount of proteolytic (caseinolytic) activity at pH optima 8.0. To investigate the type of protease in dry seeds, the class-specific inhibitors on endoproteolytic activities of crude extract was tested, and results were presented in Table 1. Pepstatin (an inhibitor of aspartic proteinase), phenyl methyl sulfonylfluoride (PMSF; an inhibitor of cysteine/serine proteinase), diisopropylphosphofluoridate (DISF; an inhibitor of serine proteinase) did not affect the activity of endoprotease. The inhibitory effect of metal chelators was also tested. While 1,10-phenanthroline strongly inhibited the endoprotease activity, ethylenediaminetetraacetic acid (EDTA) had no effect. Further, to identify the type of metal cofactor required for protease activity, it was tested by using different metal salts to overcome inhibitory effect of 1,10-

Table 1 Effect of class specific inhibitors on the activity of proteases in dry seeds of grass pea.

Inhibitor	Concentration (mM)	Protease activity (%)
No inhibitor	–	100
Pepstatin – A	10	96
PMSF	10	98
DISF	10	95
EDTA	10	100
1,10-Phenanthroline	10	0

The crude enzyme extract (1 ml) was preincubated in buffer (1 ml) and at indicated concentrations of inhibitors (0.1 ml). Caseinolytic activity was assayed as described in the "Materials and Methods" section. The activity is expressed as percentage of control—12.5 U/g

phenanthroline, and the results were depicted in Table 2. The inhibition of 1,10-phenanthroline was overcome by subsequent addition of Zn^{2+} but not Fe^{2+} or Ca^{2+} or Cu^{2+} or Mg^{2+} or Co^{2+}. These results indicate that Zn^{2+}-dependent proteases are present in dry seeds.

For further characterization of these metalloproteases in dry seed extract, we attempted PAGE in gelatin-containing gels with crude extracts with or without preincubation with class-specific protease inhibitors followed by incubation in a buffer and staining and destaining with Coomassie Brilliant Blue R-250. The extracts treated with EDTA showed three distinct proteases bands on electrophoretic separation on gelatin gel (Fig. 1). The same pattern was also shown with pepstatin-A and PMSF (not shown in figure). Conversely the enzyme extract treated with 1,10-phenanthroline did not show any protease zones, thereby indicating that at least there are three distinct active protease zones, all dependent on the presence of metal ion for their activity. These results indicated that the protease fraction consisted of three isoforms (I, II, and III) as evidenced by the appearance of three different bands on gelatin-PAGE.

Purification of Metalloprotease

To investigate any possible role of these proteases in quiescence and during germination, we also purified the metalloprotease from dry seeds of grass pea. The protease activity was measured by using the casein as substrate, absolute and specific activities calculated at each stage of purification. The results of the purification are summarized in Table 3. The crude

Table 2 Effect of different metals on protease activity in crude extracts inhibited by 1,10-Phenanthroline.

Metal ion	Concentration (mM)	Protease activity (%)
1,10-Phenanthroline	–	0
Zinc ($ZnSO_4$)	20	68
Calcium ($CaCl_2$)	20	2
Copper ($CuSO_4$)	20	5
Magnesium ($MgCl_2$)	20	8
Cobalt ($CoCl_2$)	20	0

Crude extract (1 ml) was preincubated in buffer and at 10 mM 1,10-phenanthroline (0.1 ml) for 1 h and different metal ions added at indicated concentrations and further incubated for 1 h, and caseinolytic activity was measured and the percent activity regained is presented

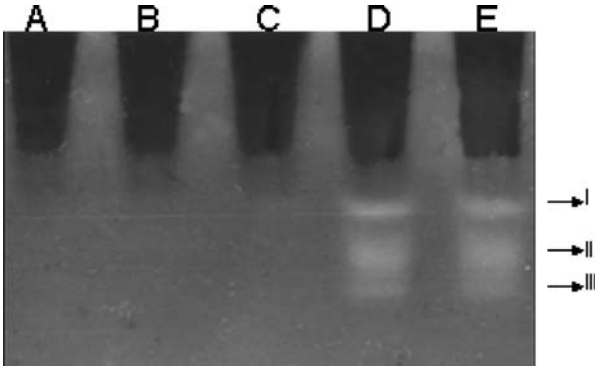

Fig. 1 Localization of endoprotease enzymes in dry seed extract by gelatin-PAGE zymography in the presence and absence of 1,10-phenanthroline and EDTA. *A–C* extracts treated with 1,10-phenanthroline, *D* extract treated with no inhibitor, *E* extract treated with EDTA enzyme extract incubated with inhibitors for 1 h in Tris–HCl buffer (0.1 M, pH 8.0) and loaded onto the gel containing 0.5% gelatin and electrophoresed at 4 C. After, electrophoresis gels were rinsed in distilled water and incubated in Tris Buffer overnight at 37 C and stained and destained

extract contains about 125 enzyme units with a specific activity of 0.038. The precipitate obtained between 20% and 40% saturation recovered nearly 86% of the enzyme activity. In the next step, the recovered enzyme passes through an affinity column of casein–alginate and eluted through KCl linear gradient. The specific activity of the enzyme is very high (4.20) and recovered 60% of the enzyme. SDS-PAGE analysis of the proteins at each step of purification shows a substantial qualitative difference between the protein profiles of crude extract (Fig. 2). Affinity chromatography separated a 3 ml fraction and was shown clearly in three low molecular weight bands. This fraction was directly subjected to gel filtration on Sephadex G-50 and observed high activity containing three distinct enzyme peaks at A_{280}. A purified enzyme fraction has been judged to be homogenous by the detection of single bands in SDS-PAGE. This is further supported by the occurrence of three individual peaks in gel filtration and a respective single band on SDS-PAGE (not shown). The molecular weight of the purified isoforms of metalloproteases was estimated to be 25, 18, and 14 kDa. The isoform II (18 kDa) has a high proteolytic activity than the other two isoforms (Table 3).

Table 3 Purification of metallo protease from the dry seeds of grass pea.

Purification step	Total protein	Total activity	Specific activity	Yield (%)	Purification fold
Crude extract	3,245	125	0.038	100	1
$(NH_4)_2SO_4$ fraction	560	108	0.192	86	5
Casein–alginate	18	76	4.220	60	110
Gel filtration					
Isoform I	9	18.9	2.100	15	55
Isoform II	5	24.0	4.800	19	126
Isoform III	3	11.8	3.920	10	102

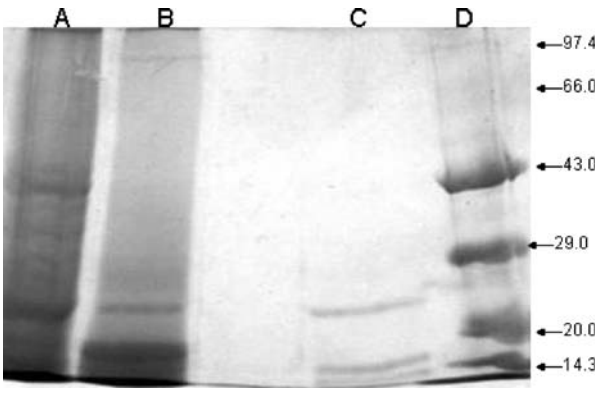

Fig. 2 Separation of purified fractions of metalloprotease from grass pea seeds on SDS-PAGE. *A* crude extract, *B* 20–40% $(NH_4)_2SO_4$ fractionation, *C* casein–alginate, *D* molecular weight markers

Discussion

Proteolytic enzymes are intricately involved in many aspects of plant physiology and development. On the one hand, they are necessary for protein turnover. Degradation of damaged, misfolded, and potentially harmful proteins provides free amino acids required for the synthesis of new proteins [2, 3]. Mobilization of seed storage proteins is an important post-germinative event in the growth and development of a seedling, and proteolytic enzymes plays a vital role in the protein mobilization process [4, 18]. In dry seeds, the existence of proteases has been demonstrated in limited species, and their exact physiological role is not known [4, 27, 28]. Numerous reviews state that the initiation of reserve protein mobilization is attributed to de novo formed endopeptidases, which, together with stored carboxypeptidases, degrade the bulk of proteins in storage organs and tissues after seeds have germinated. To study the mechanism of this physiological process, many have undertaken the task of purifying and characterizing a variety of proteases, some of which occur in germinating seeds [4, 11, 12, 15, 16, 29–33].

The reports on the presence of metalloproteases in the dry seed and during germination are very limited. The present work has demonstrated the presence of endoprotease with pH optima 8.0 in the dry seed. The endoproteolytic activity was also reported in dry seeds of horse gram [8], Indian bean [9], buckwheat [27], and vetch [34]. However, the endoprotease found in dry seeds of grass pea is the metalloprotease. This was found to be inhibited by 1,10-phenanthroline but not by EDTA and other class-specific inhibitors, thereby showing that these are metalloproteases (Table 1). Further, reactivation of the 1,10-phenanthroline, which inhibited protease by Zn^{2+}, is suggestive of the requirement of zinc for catalysis (Table 2). Since the dry seeds exhibited maximal caseinolytic activity at pH 8.0, further partial characterization of this protease was attempted by gelatin-PAGE zymography and observed three distinct zones of proteolytic activity with higher mobility. Proteolytic activity was inhibited by 1,10-phenanthroline; the in vivo inhibition of this protease was witnessed by gelatin-PAGE-zymography lending support to the metal requiring enzyme and evidenced by the appearance of three different bands on gelatin-PAGE (Fig. 1). The present work demonstrated the presence of metalloproteases specifically requiring zinc for their activity in dry seeds of grass pea. Chromatography and chromatofocusing were used by Fontanini and Jones [35] to isolate a group of metalloproteases from green malt. The metalloprotease mixture was separated by PAGE and 2-D isoelectric focusing (IEF)-PAGE on gelatin-containing gels and its individual components studied. All the metalloprotease enzymes were maximally active at pH 7–8 and were inhibited by the chelating agents EDTA and *o*-phenanthroline, but not by other class-

specific proteinase inhibitors. The activities of EDTA-inhibited enzymes were restored by the addition of low concentrations of Co^{2+}, Mn^{2+}, or Zn^{2+} ions. In nature, they probably contain Zn^{2+} ions at their active sites [21].

Although all four classes (cys, ser, metallo, and aspartic proteases) have been shown to occur in plant seeds, most described to date are cysteine proteases [3, 4, 11, 12, 16, 18, 30, 34] and have less information available on the metalloproteases and their role in protein mobilization in the dry seeds. In the present study, the three-step purification procedure yields an essentially homogenous preparation with an overall highest recovery of protease of 110%, comprising crude extract from dry seeds, $(NH_4)_2SO_4$ fractionation, and casein–alginate affinity chromatography (Table 3). The enzyme recovered in each step was judged by SDS-PAGE, and three distinct bands was observed on affinity chromatography fraction. This is further supported by the occurrence of three individual peaks in gel filtration of casein–alginate separated fraction and the respective single band on SDS-PAGE (not shown). The molecular weight of the purified isoforms of metalloproteases was estimated to be 25, 18, and 14 kDa. In barley, Wrobel and Jones [29] reported the proteases of 30, 31, 32, and 37 kDa. The protein bodies of dry buckwheat cotyledons contain a metalloproteinase as well as an aspartic proteinase and a carboxypeptidase(s) [27, 28]. The three extracellular proteases (P1, P2, and P3) were purified from *Chryseobacterium indologenes* by DEAE-Sepharose and Phenyl Sepharose chromatography and showed molecular weights of 56, 40, 40 kDa, respectively. The inhibitory effect of metal chelator EDTA and Zn-specific chelator 1,10-phenanthroline characterized all three enzymes as Zn-metalloproteases [36, 37]. The present work demonstrated the presence of three isoforms metalloproteases specifically requiring zinc for their activity in dry seeds of grass pea. Further work is under progress in the molecular characterization and identification of natural substrates within seeds and their utilization during germination of grass pea and also to enquire about the nature of the signals that initiate and regulate the process of protein mobilization.

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